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Involvement of *Manduca sexta* peptidoglycan recognition protein-1 in the recognition of bacteria and activation of prophenoloxidase system

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Abstract

Although the importance of peptidoglycan recognition proteins (PGRPs) in detecting bacteria and promoting immunity is well recognized in *Drosophila melanogaster* and other insect species, such a role has not yet been experimentally established for PGRPs in the tobacco hornworm, *Manduca sexta*. In this study, we purified *M. sexta* PGRP1 from the baculovirus-insect cell expression system, tested its association with peptidoglycans and intact bacteria, and explored its possible link with the prophenoloxidase activation system in larval hemolymph. Sequence comparison suggested that PGRP1 is not an amidase and lacks residues for interacting with the carboxyl group of *meso*-diaminopimelic acid-peptidoglycans (DAP-PGs). *M. sexta* PGRP1 gene was constitutively expressed at a low level in fat body, and the mRNA concentration became much higher after an injection of *Escherichia coli*. Consistently, the protein concentration in larval plasma increased in a time-dependent manner after the immune challenge. Purified recombinant PGRP1 specifically bound to soluble DAP-PG of *E. coli* but not to soluble Lys-type PG of *Staphylococcus aureus*. In addition, this recognition protein completely bound to insoluble PGs from *Micrococcus luteus*, *Bacillus megaterium* and *Bacillus subtilis*, whereas its association with the bacterial cells was low even though their peptidoglycans are exposed on the cell surface. After PGRP1 had been added to plasma of naïve larvae in the absence of microbial elicitor, there was a concentration-dependent increase in prophenoloxidase activation. Phenoloxidase activity, as usual, increased after the plasma was incubated with peptidoglyans or bacterial cells. These increases became more prominent when insoluble *M. luteus* or *B. megaterium* PG or soluble *E. coli* PG and PGRP1 were both present. Statistic analysis suggested a synergistic effect caused by interaction between PGRP1 and these PGs. Taken together, these results indicated that PGRP1 is a member of the *M. sexta* prophenoloxidase activation system, which recognizes peptidoglycans from certain bacteria and initiates the host defense response. The unexplained difference between the purified PGs and intact bacteria clearly reflects our general lack of understanding of PGRP1-mediated recognition and how it leads to proPO activation.

Keywords

pattern recognition; hemolymph protein; melanization; insect immunity; serine protease pathway

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1. Introduction

Arthropods rely solely on their innate immunity to fight off invading pathogens (Gillespie et al., 1997; Lavine and Strand, 2002; Lemaitre and Hoffmann, 2007). This system is elicited by conserved molecular patterns associated with microbes, such as peptidoglycan (PG), lipopolysaccharide, and β-1,3-glucan. Peptidoglycan, a cell wall component of bacteria, is a high molecular weight polymer consisting of unbranched glycan strands cross-linked by short stem peptides (Vollmer et al., 2008). The glycan strands are composed of alternating β-1,4-linked *N*-acetylglucosamine and *N*-acetylmuramic acid. The stem peptides attached to *N*-acetylmuramic acid residues contain a lysine or *meso*-diaminopimelic acid (DAP) at the third position, which connects these peptides directly or indirectly through a peptide cross-bridge. Lys-type PGs are found in most Gram-positive bacteria, whereas DAP-PGs are present in Gram-negative bacteria and the Gram-positive genera *Bacillus* and *Clostridium*. Different bacteria vary in stem peptide, cross-bridge, and physical properties (*e.g*. thickness, elasticity, porosity) of their peptidoglycans.

Peptidoglycan recognition proteins (PGRPs) are immunity-related molecules conserved from insects to humans (Royet and Dziarski, 2007; Chaput and Boneca, 2007). The founding member of this protein family was isolated from the silkworm, *Bombyx mori* (Yoshida et al., 1996). The carboxyl-terminal PG-binding domain of *B. mori* PGRP is approximately 165 residues long and homologous to lysozyme of bacteriophage T7 (Ochiai and Ashida, 1999; Kang et al., 1998). Due to the lack of key catalytic residues in T7 lysozyme, the silkworm protein does not cleave an amide bond between *N*-acetylmuramic acid and L-alanine of the stem peptide. Its binding of Lys-type PG from *Micrococcus luteus*, however, triggers a serine proteinase cascade that leads to prophenoloxidase (proPO) activation and melanin formation. In *Drosophila*, PGRP-SA and -SD bind Lys-PG to induce Toll pathway; PGRP-SA is also involved in proPO activation; PGRP-LC and -LE bind DAP-PG fragments and induce Imd pathway; PGRP-LC stimulates phagocytosis (Charroux et al., 2009). On the other hand, *Drosophila* PGRP-SB, -SC, and -LB hydrolyze the amide bond between *N*-acetylmuramic acid and L-alanine in the stem peptide to reduce the amount of immunogenic muropeptide and down-regulate the immune responses (Royet and Dziarski, 2007). In *Tenebrio molitor*, clustered PGRP-SA detects Lys-type PG and activates the proPO cascade and Toll pathway (Park et al., 2007).

Three-dimensional structures of PG-binding domains in the *Drosophila* and human PGRPs reveal an α/β mixed fold similar to that of T7 lysozyme (Kim et al., 2003; Reiser et al., 2004; Guan et al., 2004 and 2005; Lim et al., 2006; Cho et al., 2007; Leone et al., 2008). This general fold consists of a central β-sheet and three peripheral α-helices, parts of which form the walls and bottom of a PG-binding groove found in most PGRPs. The groove is composed of His, Tyr, His, Thr and Cys/Ser residues that correspond to His17, Tyr46, His122, Lys128 and Cys130 of T7 lysozyme (Mellroth et al., 2003). Some of these residues are responsible for Zn^{2+}-binding and *N*-acetylmuramoyl-L-alanine amidase activity of T7 lysozyme and hydrolytic PGRPs. Structural analysis of free or muropeptide-bound PGRPs, along with site-directed mutagenesis and binding assays, suggests that certain residues in the binding groove are responsible for the differential recognition of Lys- and DAP-PGs (Guan et al., 2004; Lim et al., 2006; Cho et al., 2007).

In *M. sexta*, cDNA cloning disclosed a 19 kDa protein most similar in sequence to *B. mori* PGRP (identity: 54%) (Yu et al., 2002; Zhu et al., 2003). While its protein level in larval plasma increased after an injection of *M. luteus*, immunological function of *M. sexta* PGRP1 remains unclear. In fact, unpublished data indicated that addition of PGRP1 to plasma failed to enhance melanization triggered by *M. luteus* (Kanost et al., 2004). To explore its possible role in bacteria recognition and initiation of proPO activation, we expressed *M. sexta* PGRP1 in insect cells

and tested its binding to soluble and insoluble PGs from various bacteria and to intact bacterial cells. By correlating the binding data with proPO activation upon exposure to these elicitors, we confirmed the unusual binding properties of *M. sexta* PGRP1 and established its role as a sensor of the proPO activation system. Implications of our findings regarding recognition of bacteria are discussed as well.

2. Materials and methods

2.1. Insect rearing, bacterial challenge, and hemolymph collection

M. sexta eggs were purchased from Carolina Biological Supply and larvae were reared on an artificial diet (Dunn and Drake, 1983). Day 2, 5^{th} instar larvae were injected with formaldehyde-killed *Escherichia coli* (2×10^8 cells/larvae). Hemolymph was collected from a cut proleg of the immune challenged larvae 6, 12, and 24 h later. For use as a control at 0 h, hemolymph was also collected from day 2, 5^{th} instar naïve larvae. After centrifugation at 5000*g* for 5 min, plasma samples from the naïve and induced insects were aliquoted and stored at −80 °C.

2.2. Detection of PGRP1 in hemolymph

The control and induced plasma samples were analyzed by mixing 2 μl of plasma with 6 μl of 20 mM Tris-HCl, pH 8.0 and 4 μl of 5 × SDS sample buffer. After incubation at 95 °C for 5 min, 7 μl of the mixture was separated by 15% SDS-PAGE, transferred to a nitrocellulose membrane, and reacted with 1:2000 diluted PGRP1 polyclonal antiserum kindly provided by Dr. Michael Kanost at Kansas State University. Antibody-antigen complexes were detected using alkaline phosphatase conjugated to goat-anti-rabbit IgG, 5-bromo-4-chloro-3-indolyl phosphate, and nitro-blue tetrazolium (Bio-Rad).

2.3. Reverse transcription-PCR analysis of PGRP1 mRNA levels

Hemocyte and fat body total RNA was prepared from the naïve and induced 5^{th} instar larvae (day 3) using Micro-to-Midi Total RNA Purification System (Life Technologies). The RNA (2 μg), oligo(dT) (0.5 μg) and dNTPs (1 μl, 10 mM each) were mixed with diethylpyrocarbonate-treated H_2O in a final volume of 12 μl, denatured at 65 °C for 5 min, and quickly chilled on ice for 3 min. M-MLV reverse transcriptase (1 μl, 200 U/μl) (Life Technologies), 5 × buffer (4 μl), 0.1 M dithiothreitol (2 μl), and RNase OUT (1 μl, 40 U/μl) (Life Technologies) were added to the denatured RNA sample (12 μl) for first strand cDNA synthesis at 37 °C for 50 min. *M. sexta* ribosomal protein S3 mRNA was used as an internal control to normalize the cDNA samples using specific primers j501 (5′-GCCGTTCTTGCCCTGTT) and j504 (5′-CGCGAGTTGACTTCGGT). Primers j297 (5′-CACATTGAGTACCTTACCCGTCCA) and j298 (5′-GAACGAAGATCCGATGTCCAGTC) were used to amplify *M. sexta* PGRP1 cDNA under conditions empirically chosen to avoid saturation: 30 cycles of 94 °C, 30 s; 50 °C, 30 s; 72 °C, 30 s in a duplex PCR reaction. Relative levels of PGRP1 mRNA in the samples were estimated by 1.5% agarose gel electrophoresis.

2.4. Construction of recombinant baculovirus for PGRP1 expression

M. sexta PGRP1 cDNA (obtained from Dr. Michael Kanost at Kansas State University) was amplified using PCR primer j285 (5′-GGAATTCACTGCAACGTCGTC) and j288 (5′-CTCGAGGTCTTTATATTCGGACAC). The PCR product was cloned into pGEM-T (Promega) and completely verified by DNA sequencing. The 528 bp *Eco*RI-*Xho*I fragment was retrieved from the plasmid by restriction digestion and directionally inserted into the same sites of $pMFH_6$ (Lu and Jiang, 2008) to generate plasmid PGRP1/$pMFH_6$, which allows efficient secretion of the recombinant protein into medium using the honeybee mellitin signal

peptide and purification on a Ni^{2+}-nitrilotriacetic (NTA) agarose column via the carboxyl-terminal hexahistidine tag. *In vivo* transposition of the expression cassette, selection of bacterial colonies carrying the recombinant bacmid, and isolation of bacmid DNA were carried out according to manufacturer's protocols (Life Technologies). The initial viral stock (V_0) was obtained by transfecting *Spodoptera frugiperda Sf*9 cells with a bacmid-CellFECTIN mixture, and its titer was improved through serial infections. The V_6 viral stock, containing the highest level of baculovirus (1~2 × 10^8 pfu/ml), was stored at −70 °C for further experiments.

2.5. Expression and purification of M. sexta PGRP1

*Sf*9 cells (2.0 × 10^6 per ml) in 100 ml of Sf-900™ III serum-free medium (Life Technologies) were infected with the baculovirus stock at a multiplicity of infection of 5~10 and grown at 27 °C for 96 h with gentle agitation (100 rpm). After centrifugation at 5000*g* for 10 min, the culture supernatant was collected and mixed with an equal volume of distilled water and once again centrifuged at 22100*g* for 20 min. The cleared supernatant was applied to a dextran sulfate-Sepharose column (5 ml) equilibrated with buffer A (1 mM benzamidine in 10 mM potassium phosphate, pH 6.4). Following a washing step with 25 ml of buffer A, bound proteins were eluted with a linear gradient of 0–1.0 M NaCl in buffer A (30 ml). Based on the result of immunoblot blot analysis, fractions containing *M. sexta* PGRP1 were pooled and applied to a Ni^{2+}-NTA agarose column (1 ml) (Qiagen), equilibrated with buffer B (50 mM potassium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 0.005% Tween-20). After washing with buffer B (5 ml), bound proteins were eluted from the column with a linear gradient of 10–100 mM imidazole in buffer B (20 ml). Finally, tightly bound proteins were eluted with 5 ml of buffer B containing 250 mM imidazole. All the purification steps were carried out at 4 °C. After electrophoretic analysis, PGRP1 fractions were combined and concentrated using Amicon ultracentrifugal 5K MWCO filter device (Millipore). Buffer exchange with 20 mM Tris-HCl, pH 7.5, 50 mM NaCl was performed on the same device.

2.6. Plate assay of PGRP1 binding to soluble peptidoglycans

Soluble PGs (InvivoGen) from *E. coli* and *Staphylococcus aureus* were used as ligands in a binding assay. Each ligand (2 μg/well) was applied to wells in a 96-well microplate, air dried overnight at room temperature, and fixed to the wells at 60 °C for 30 min. After blocking with 200 μl of 1 mg/ml bovine serum albumin (BSA) in Tris buffered saline (TBS: 137 mM NaCl, 3 mM KCl, 25 mM Tris-HCl, pH 7.6) at 37 °C for 2 h and after washing with 200 μl TBS four times (5 min each), PGRP1 (300 ng) in 50 μl TBS containing 0.1 mg/ml BSA was added to the wells and incubated at room temperature for 3 h. To test binding specificity, a competition experiment was performed in parallel by pre-incubating PGRP1 (300 ng) with 200 μg of the ligand at room temperature for 30 min. The mixture was then added to the ligand-coated wells and incubated at room temperature for 3 h. Following a washing step with TBS, 100 μl of 1:1000 diluted anti-$(His)_5$ monoclonal antibody (Bio-Rad) in TBS containing 0.1 mg/ml BSA was added to the wells and incubated at 37 °C for 2 h. After washing with 200 μl of TBS four times, 100 μl of 1:1500 diluted goat-anti-mouse IgG conjugated to alkaline phosphatase (Bio-Rad) in TBS containing 0.1 mg/ml BSA was added to the wells and incubated at 37 °C for 2 h. After washing with TBS four times and 0.5 mM $MgCl_2$, 10 mM diethanolamine once, 50 μl aliquots of *p*-nitrophenyl phosphate (1.0 mg/ml in the diethanolamine buffer) were added to the wells and absorbance at 405 nm was monitored in the kinetic mode on a VersaMax microplate reader (Molecular Devices).

2.7. Isolation of insoluble peptidoglycans from Gram-positive bacteria

M. luteus, *S. aureus*, *Bacillus megaterium*, and *Bacillus subtilis* were separately grown in Luria-Bertani medium (2.0 L) overnight at 37 °C with shaking. These bacteria, separated from the medium by centrifugation at 2000*g* for 20 min, were resuspended in 100 ml saline (0.85%

NaCl) and heated at 100 °C for 20 min. The cells were washed twice with saline (50 ml each), once with water (50 ml), and three times with acetone (50 ml each) by complete resuspension and centrifugation. After drying at 37 °C for 8 h, peptidoglycans were extracted from the bacteria according to a modified protocol (Tsuchiya et al., 1996). Briefly, dried cells (10 g) were stirred in 300 ml of 10% trichloroacetic acid in a boiling water bath for 20 min. After centrifugation at 10000*g* for 30 min, the pellet was washed three times with 250 ml water and once with 250 ml of buffer C (100 mM Tris-HCl, pH 7.5, 20 mM $MgCl_2$, 1 mM $CaCl_2$). Bacteria resuspended in 100 ml buffer C were incubated with 30 mg of bovine trypsin at 37 °C for 24 h with gentle agitation. After the enzyme was inactivated with 1 mM phenylmethanesulfonyl fluoride at 37 °C for 30 min, treated cells were centrifuged at 10000*g* for 30 min and washed with 200 ml of H_2O ten times. Each time the precipitate was completely resuspended by sonication and, after the final wash, lyophilized and stored at −20 °C.

2.8. Binding of PGRP1 to insoluble peptidoglycans

One mg of insoluble PG was mixed with 10 μl of PGRP1 (3 μg) and 40 μl of buffer D (20 mM Tris-HCl, pH 8.0, 20 mM NaCl). After incubation for 2 h at 4 °C with mixing, the mixture was centrifuged at 16000*g* for 15 min. The supernatant was treated with 5 × SDS sample buffer and analyzed as unbound fraction. The pellet was washed 3 times with 200 μl of buffer D each and then boiled with 20 μl of 2 × SDS sample buffer for 5 min to obtain bound fraction. The total, unbound, and bound samples (equivalent to 7 μl of 1:5 diluted PGRP1) were separated by 15% SDS-PAGE followed by immunoblot analysis using 1:2000 diluted anti-$(His)_5$ monoclonal antibody and goat-anti-mouse IgG conjugated to alkaline phosphatase.

2.9. Binding of PGRP1 to bacterial cells

A single bacterial colony was grown in LB medium overnight at 37 °C and subcultured in 4 ml of the same medium until OD_{600} was close to 0.8 (*ca*. 1.6×10^8 cells/ml). After centrifugation at 1000*g* and washing with buffer D twice, the bacteria were resuspended in 40 μl of buffer D. Purified PGRP1 (10 μl, 3 μg) was added to the cell suspension and incubated for 2 h at 4 °C with mixing. After centrifugation at 4000*g* for 15 min, the supernatant was treated with 5 × SDS sample buffer and analyzed as unbound fraction. The cell pellet was washed 3 times with 200 μl of buffer D, suspended with 20 μl of 2 × SDS sample buffer, and heated at 95 °C for 5 min to obtain the bound fraction. The total, unbound, and bound fractions (equivalent to 7 μl of 1:5 diluted PGRP1) were analyzed as described in Section 2.8.

2.10. Role of PGRP1 in proPO activation

Plasma from day 3, fifth instar naïve larvae was diluted ten times with buffer E (20 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$, 0.001% Tween-20). Five μl of diluted sample was mixed with 19 μl of buffer E and incubated at 25 °C for 10 min. PO activity was determined using dopamine as a substrate on a microplate reader (Jiang et al., 2003). Plasma samples with low PO activity were pooled and stored in 10 μl aliquots at −80 °C. Aliquots of the 1:10 diluted plasma (5 μl) with buffer E (1 μl) or purified recombinant PGRP1 (1 μl, 0.2 μg/μl) were separately incubated with 1 μl of buffer E (negative control) or one of the following elicitors to investigate PGRP1-enhanced proPO activation: insoluble PGs from *M. luteus*, *S. aureus*, *B. megaterium* and *B. subtilis* (1 μg), soluble PGs (1 μg) from *S. aureus* and *E. coli*, curdlan (10 μg), and 2×10^5 cells of *M. luteus*, *S. aureus*, *B. megaterium*, *B. subtilis* and *E. coli*. The controls were mixtures of diluted plasma with buffer E, elicitor, or PGRP1, and the test was diluted plasma mixed with elicitor and PGRP1. The total volume of the four mixtures in each elicitor group was adjusted to 24 μl with buffer E and incubated at 25 °C for 1 h prior to the PO activity assay.

3. Results

3.1. Structure features, expression pattern, and recombinant production of M. sexta PGRP1

M. sexta PGRP1 was initially identified as a bacteria-induced pattern recognition receptor in plasma (Yu et al., 2002; Zhu et al., 2003). The open reading frame encodes a 192-residue protein sequence, including a 20-residue signal peptide. The mature protein has a calculated molecular mass of 19,319 Da. No potential *N*- or *O*-linked glycosylation sites are found in the sequence. The conserved PGRP domain is located between residues 22–164. Sequence alignment with other PGRPs revealed that *M. sexta* PGRP1 has a serine residue (Ser148) in the position equivalent to Cys130 in T7 lysozyme (Fig. 1). This common feature of receptor-type PGRPs suggests that *M. sexta* PGRP1 is not an amidase (Mellroth et al., 2003). Asn60 and Tyr61of *M. sexta* PGRP1 correspond to Asn and Phe of human PGRP-1αC, Asp and Phe of *Drosophila* PGRP-SA, as well as Gly and Trp of *Drosophila* PGRP-LC and -LE (Fig. 1). The Asn/Asp-Phe and Gly-Trp are responsible for specific binding to Lys- and DAP-type PGs, respectively (Guan et al., 2004; Kaneko et al., 2004). Additionally, Ser80 of *M. sexta* PGRP1 is in a position equivalent to Arg254 in *Drosophila* PGRP-LE, which interacts with the carboxyl group of DAP-PGs (Fig. 1) (Lim et al., 2006). This Arg residue is conserved in all known *Drosophila* and human PGRPs that recognize DAP-PGs, but not always found in PGRPs that bind Lys-PGs (Onoe et al., 2007).

The PGRP1 gene was constitutively expressed at a low level in fat body, and its transcripts became more abundant after an immune challenge with *E. coli* (Fig. 2). The mRNA level was much lower in hemocytes from naïve larvae, which also increased after the bacteria injection. The PGRP1 protein is present in the plasma of naïve larvae and its concentration dramatically increased at 12 h after the injection of *E. coli*. These data indicated that constitutive expression of *M. sexta* PGRP1 is induced in a time-dependent manner by *E. coli*, as well as by *M. luteus* in the previous experiments (Yu et al., 2002;Zhu et al., 2003).

We expressed *M. sexta* PGRP1 in a baculovirus-insect cell system. The recombinant protein in the medium reached the highest level at 72–84 h after infection. PGRP1 was directly captured by cation exchange chromatography on a dextran sulfate-Sepharose column and further purified by affinity chromatography on a Ni-NTA column. From 100 mL conditioned medium, we obtained 0.15 mg of PGRP1. The purified recombinant protein migrated as a single band at 19 kDa position, which was recognized by anti-$(His)_5$ and PGRP1 antibodies (Fig. 3).

3.2. Differential binding of M. sexta PGRP1 to microbial cells and cell wall components

To measure the relative binding of PGRPs to Lys- and DAP-PGs, we developed an ELISA-based, semiquantitative binding assay. Contrary to the prediction based on sequence comparison (Fig. 1), this test showed that *M. sexta* PGRP1 binds soluble DAP-PG from *E. coli* (Fig. 4A) more than soluble Lys-PG from *S. aureus* (Fig. 4B). The binding was specific for the former since pre-incubation of PGRP1 with excess amount of *E. coli* PG eliminated its binding to the ligand immobilized on the ELISA plate. In contrast, the association of PGRP1 with Lys-PG of *S. aureus* was nonspecific, as shown by the competition experiment. The total binding was not significantly higher than the negative control of BSA.

M. sexta PGRP1 showed complete binding to insoluble PGs from *M. luteus* (Lys-type), *B. megaterium* (DAP-type), and *B. subtilis* (DAP-type) (Fig. 5, A, E, and G). Consistent with the ELISA results, recombinant PGRP1 did not show any binding to insoluble PG from *S. aureus* (Lys-type) (Fig. 5C). When PGRP1 was incubated with *M. luteus*, *S. aureus*, *B. megaterium* and *B. subtilis* cells (Fig. 5, B, D, F, and H), partial binding was observed in each case. Although PGs are located on the surface of Gram-positive bacteria and enough cells were used, a majority of PGRP1 was detected in the flow-through fractions, indicating that other

cell surface structures (*e.g*. techoic acids, lipoteichoic acids, and proteins) may interfere with the binding. In contrast, low complexity and high accessibility of insoluble PGs purified from *M. luteus*, *B. megaterium*, and *B. subtilis* may have contributed to the complete binding.

3.3. Role of M. sexta PGRP1 in the proPO activation system

To test a possible role of *M. sexta* PGRP1 in the proPO activation system, we added purified PGRP1 to the cell-free hemolymph from naïve larvae and observed a concentration-dependent increase in proPO activation in the absence of bacterial elicitor (Fig. 6). In contrast, no such response was detected after we added BSA to the plasma.

How may exogenous PGRP1 enhance proPO activation triggered by various PGs? To answer this question, we incubated diluted plasma with PGs in the absence or presence of recombinant PGRP1. Compared with the control of plasma only, small but significant increase (#2 - #1) was observed after insoluble PG from *M. luteus*, *B. megaterium*, or *B. subtilis* (Fig. 7, A, D and E) or curdlan (Fig. S1) was added, indicating that the proPO activation system was functional. Such increase (#2 - #1, representing PG-plasma interaction) was not as large as that in the mixtures of plasma and PGRP1 (#3 - #1, representing exogenous PGRP1-plasma interaction). When PG, PGRP1, and diluted plasma were present simultaneously, the PO activity increase (#4 - #1) was significantly higher than the sum of the components [(#2 – #1) + (#3 – #1)] in the following three cases: insoluble PG from *M. luteus* (Lys-type) and *B. megaterium* (DAP-type) and soluble PG from *E. coli* (DAP-type) (Fig. 7, A, D, and F). This synergistic effect, likely caused by specific interaction of these PGs and exogenous PGRP1 (Fig. 4A and Fig. 5, A, E, and G), was not observed in the #4 reactions containing insoluble or soluble PG from *S. aureus* (Fig. 7, B and C). Consistent with that, the recombinant PGRP1 did not bind to *S. aureus* PGs (Fig. 4B and Fig. 5C). Neither did *M. sexta* PGRP1 synergistically enhance proPO activation triggered by curdlan (Fig. S1), an insoluble form of β-1,3-glucan not binding to PGRP1 (data not shown). Insoluble PG from *B. subtilis* (DAP-type) did cause an increase in proPO activation (#4 - #1), but it was statistically insignificantly higher than the sum of (#2 – #1) and (#3 – #1) (Fig. 7E).

We have further examined a possible synergistic enhancement of proPO activation caused by interaction between exogenous PGRP1 and whole bacteria. *M. luteus*, *S. aureus*, *B. megaterium*, *B. subtilis*, and *E. coli* cells all stimulated the melanization response by interacting with plasma factors (Fig. 8, #2 vs. #1). The elevated PO activity was comparable to that caused by exogenous PGRP1 interacting with plasma proteins (#2 vs. #3). After bacteria, PGRP1, and plasma had been incubated for 1 h at 25 °C, the PO activity increase (#4 - #1) was lower than the sum of the two components (#2 + #3 – 2×#1). The lack of synergism coincided with the incomplete binding of PGRP1 to these bacteria (Fig. 6).

4. Discussion

Insects have evolved a battery of proteins to recognize characteristic molecular patterns of microbes and promote defense responses (Gillespie and Kanost, 1997; Steiner et al., 2004). In *M. sexta*, immulectins, β-1,3-glucan recognition proteins, and other plasma factors participate in the immune surveillance (Jiang, 2008; Ragan et al., 2008). *M. sexta* PGRP1 is a hemolymph protein induced upon bacterial injection (Yu et al., 2002; Zhu et al., 2003). Although it is 54% identical in sequence to the *B. mori* PGRP (a sensor of the silkworm proPO activation cascade), addition of *M. sexta* PGRP1 to plasma failed to enhance proPO activation stimulated by *M. luteus* cells (Kanost et al., 2004). Knockdown of PGRP1 expression did not have any effect on cellular immunity but increased the susceptibility of larvae to *Photorhabdus asymbiotica* (Eleftherianos et al., 2007). To elucidate its immunological function, we carried out this study using recombinant PGRP1 and a variety of microbial elicitors. The biochemical analysis

provided evidence that *M. sexta* PGRP1 functions as a recognition molecule for Lys-PG of *M. luteus* and DAP-PGs and that the specific binding to PGs triggers the proPO activation cascade.

4.1. Differences in binding to Lys-PGs from M. luteus and S. aureus

M. sexta PGRP1 bound insoluble Lys-PG of *M. luteus* (Fig. 5A) but not that of *S. aureus* (Fig. 5C). Nor did specific binding occur after the protein was incubated with soluble PG of *S. aureus* (Fig. 4B). This is consistent with the observation that *M. sexta* PGRP1 in induced plasma did not bind to *S. aureus* cells (Ragan, 2008) although, in the absence of other proteins, the recombinant PGRP1 slightly associated with the bacteria (Fig. 5D). The lack of binding also coincided with the result that PGRP1 did not further enhance proPO activation triggered by *S. aureus* cells or its PG (Fig. 7, B and C;Fig. 8B). Similarly, *Drosophila* PGRP-LC mediates the IMD pathway activation by *M. luteus* PG but not by *S. aureus* PG (Kaneko et al., 2005). *S. aureus* PG is highly cross-linked by a pentaglycine bridge, whereas *M. luteus* uses a branched pentapeptide (identical to its stem-peptide) for the cross-bridge (Shokman and Barrett, 1983). The bridge structure, low cross-linking, and high accessibility of glycan strand of *M. luteus* PG appear to be responsible for the PGRP binding and proPO activation. *S. cynthia ricini* PGRP-A binds to linear *M. luteus* PG with high affinity, but little binding was detected with cross-linked PG from the same species (Onoe et al., 2007).

4.2. Binding to DAP-PGs from E. coli, B. megaterium, and B. subtilis

M. sexta PGRP1 also recognized insoluble DAP-PGs of *B. megaterium* and *B. subtilis* (Fig. 5, E and G). While DAP-PGs are exposed on the surface of Gram-positive genera *Bacillus* and *Clostridium*, they are located underneath the outer membrane of Gram-negative bacteria. Thus, we tested soluble DAP-PG purified from *E. coli* and detected specific binding (Fig. 4A). PGRP-A of the eri-silkworm has a similar specificity by binding to *Bacillus licheniformis* and *M. luteus* PGs (Onoe et al., 2007). In *Drosophila*, recombinant PGRP-SA, -SC1b, -LC, and -LF bind to both Lys- and DAP-PGs *in vitro* (Mellroth et al., 2003 and 2005; Persson et al., 2007).

The modification of glycan strand, composition of stem peptide, type and extent of cross-linking, and interference of other cell surface components all seem to affect associations between PGs and their recognition proteins (Royet and Dziarski, 2007). *M. sexta* PGRP1 binds to one milligram of insoluble DAP-PGs (equivalent to those in 1.0×10^9 bacteria) far more completely than to 6.4×10^8 intact cells (Fig. 5, E through H) and the binding to *B. megaterium* cells was more complete than to *B. subtilis*, probably due to a lower degree of cross-linking in *B. megaterium* PG (Bricas et al., 1967) and deacetylation of glucosmine in *B. subtilis* PG (Atrih et al., 1999). These structural differences may also be responsible for the differential stimulation of melanization by PGs from the two *Bacillus* species (Fig. 7, D and E).

In the beetle *Holotrichia diomphalia*, PGRP binds to β-1,3-glucan (a polysaccharide from fungal cell wall) and activates proPO system (Lee et al., 2004). To test if *M. sexta* PGRP1 does that, we incubated larval plasma with curdlan in the absence or presence of the recombinant protein. The coexistence of insoluble β-1,3-glucan and PGRP1 failed to synergistically enhance proPO activation (Fig. S1). This result is consistent with the lack of curdlan binding by *M. sexta* PGRP1 (data not shown).

4.3. Structural basis for the binding specificity of M. sexta PGRP1

Both mammalian and insect PGRPs contain about sixteen residues that contact PG (Guan et al., 2004). Five of them (His208, His231, Tyr242, His264, and Asn269 in human PGRP-1αC) form a nearly contiguous patch on the groove floor, which correspond to His32, His55, Ser66, Pro88 and Asn93 in *M. sexta* PGRP1 (Fig. 1). Asn236 and Phe237 of human PGRP-1αC form

several van der Waals bonds with the side chain of lysine in Lys-PG (Guan et al., 2004). Sequence variations at these positions affect the ability of some PGRPs to discriminate Lys- and DAP-PGs (Steiner, 2004; Royet and Dziarski, 2007; Charroux et al., 2009). For instance, *Drosophila* PGRP-SA which recognizes Lys-PG contains Asp96 and Phe97 at the two sites (Fig. 1). In contrast, *Drosophila* PGRP-LC and -LE which recognize DAP-PG have Gly and Trp residing at the corresponding positions (Kaneko et al., 2004). *M. sexta* PGRP1 and *Trichoplusia ni* PGRP, with Asn60 and Tyr61 located in the same sites (Fig. 1), recognize Lys-PG from *M. luteus* and DAP-PG from *B. megaterium* (Kang et al., 1998). It has also been suggested Arg254 in *Drosophila* PGRP-LE interacts with the carboxyl group of DAP-PG (Lim et al., 2006). This Arg is conserved in *Drosophila* and human PGRPs which recognize DAP-PG, but not always present in PGRPs that recognize Lys-PG. *M. sexta* PGRP1 and *S. cynthia* PGRP-A have a serine residue at the corresponding position (Fig. 1). Since both PGRPs recognize Lys- and DAP-type PGs (Onoe et al., 2007), this Arg residue seems dispensable for binding to DAP-PG.

4.4. Recognition of PGs by PGRP1 and stimulation of proPO activation

A connection between PGRP1 and the proPO cascade is supported by the observation that adding PGRP1 to the larval plasma concentration-dependently enhanced proPO activation in the absence of a microbial elicitor (Fig. 6). An increase in spontaneous melanization was observed when PGRP-LE was over-expressed in *Drosophila* (Takehana et al., 2002). Constitutively present in hemolymph at a low level (Fig. 2), PGRPs probably form clusters only on pathogen surface and transmit the invasion signal to other molecules (Park et al., 2007). Supplementation of recombinant PGRPs increased their plasma concentrations and may have triggered pathogen-independent self-association of the pattern recognition molecules, a process favoring interactions with other components of the proPO activation system.

The correlation between binding characteristics of *M. sexta* PGRP1 and proPO activation further supported the protein's role as a biosensor of the cascade (Table 1). There was a complete binding of *M. sexta* PGRP1 to three insoluble PGs: *M. luteus* and *B. megaterium* PGs interacted with exogenous PGRP1 and synergistically enhanced proPO activation; a similar increase caused by *B. subtilis* PG was statistically insignificant. When purified PGs were replaced by bacteria in these reactions, we did not observe any synergistic enhancement. This is consistent with the previous finding that supplementation of PGRP1 to larval plasma did not boost proPO activation after exposure to *M. luteus* (Kanost et al., 2004;Ragan, 2008). The complex structure of cell surface may have interfered the binding of PGRP1 to the bacteria. PGRP1 did not bind to soluble PG of *S. aureus* or enhance proPO activation triggered by *S. aureus*. In contrast, specific binding of PGRP1 to soluble PG isolated from *E. coli* led to a synergistic increase in PO activity.

4.5. Concluding remarks

We have produced *M. sexta* PGRP1 as a soluble 19 kDa protein in insect cells and confirmed its induced expression in larvae. The recombinant protein specifically binds to Lys-PG from *M. luteus* and DAP-PGs from Gram–negative bacteria (*e.g. E. coli*) and Gram-positive *Bacilli*. This unusual binding specificity is probably related to Asn60, Tyr61 and Ser80 in the protein. The recognition is affected by glycan structure, stem peptide, and cross-linking of PGs, as well as by other cell wall components. Significant and specific binding of PGs by PGRP1 correlates with synergistic enhancement in the proteolytic activation of proPO in plasma. PGRP1 is a component of the *M. sexta* proPO system, but we do not understand precisely how PG-PGRP1 recognition leads to proPO activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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The abbreviations used are

PG and PGRP	peptidoglycan and its recognition protein
DAP	*meso*-diaminopimelic acid
PO and proPO	phenoloxidase and its precursor
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
BSA	bovine serum albumin, NTA, nitrilotriacetic acid
TBS	Tris buffered saline

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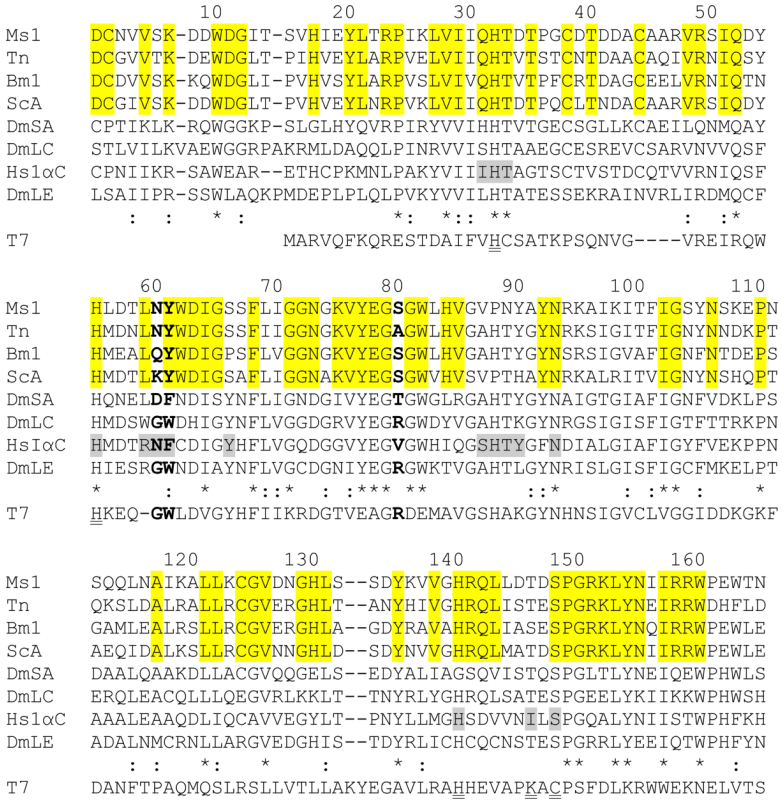

Fig. 1. Multiple sequence alignment of PGRP sequences and T7 lysozyme

Amino acid sequences of *M. sexta* PGRP1 (Ms1, AF413068), *T. ni* PGRP (Tn, AAC31820), *B. mori* PGRP (Bm1, BAA77209), *S. cynthia* PGRP-A (ScA, BAF03522), *D. melanogaster* PGRP-SA (DmSA, NP_572727), -LC (DmLC, NP_729468.2), -LE (DmLE, NP_573078), *H. sapiens* PGRP-IαC (HsIαC, AAK72484), and T7 lysozyme (T7, NP_0419731) are aligned. Residue numbers of mature *M. sexta* PGRP1 are indicated on top of its sequence. Identical and similar residues in PGRPs are marked with "*" and ":", respectively. Residues corresponding to Asn236 and Phe237 of human PGRP-IαC and Arg254 of *Drosophila* PGRP-LE are shown in bold. The muramyl tripeptide-interacting residues in human PGRP-IαC are shaded gray, residues identical in the lepidopteran PGRPs are highlighted, and residues for Zn^{2+}-binding and amidase activity in T7 lysozyme are double underlined.

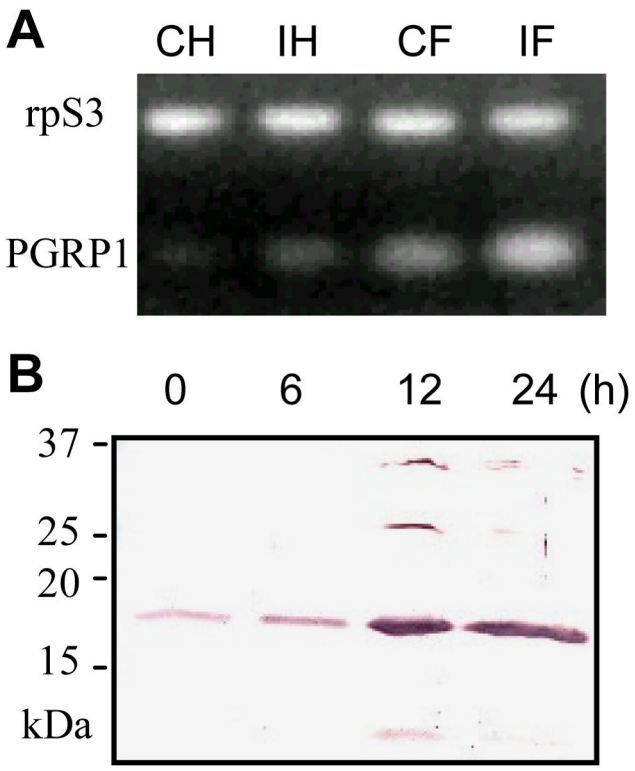

Fig. 2. Induced expression of *M. sexta* PGRP1 gene

(**A**) RT-PCR analysis of total RNA samples from control hemocytes (CH) and fat body (CF) of naïve larvae and induced hemocytes (IH) and fat body (IF) of larvae at 24 h after injection with *E. coli*. *M. sexta* ribosomal protein S3 (rpS3) transcripts were normalized for the analysis of PGRP1 mRNA. (**B**) Immunoblot analysis of plasma samples from larvae at 0, 6, 12, 24 h after injection with *E. coli*. Diluted PGRP1 antiserum was used as the first antibody.

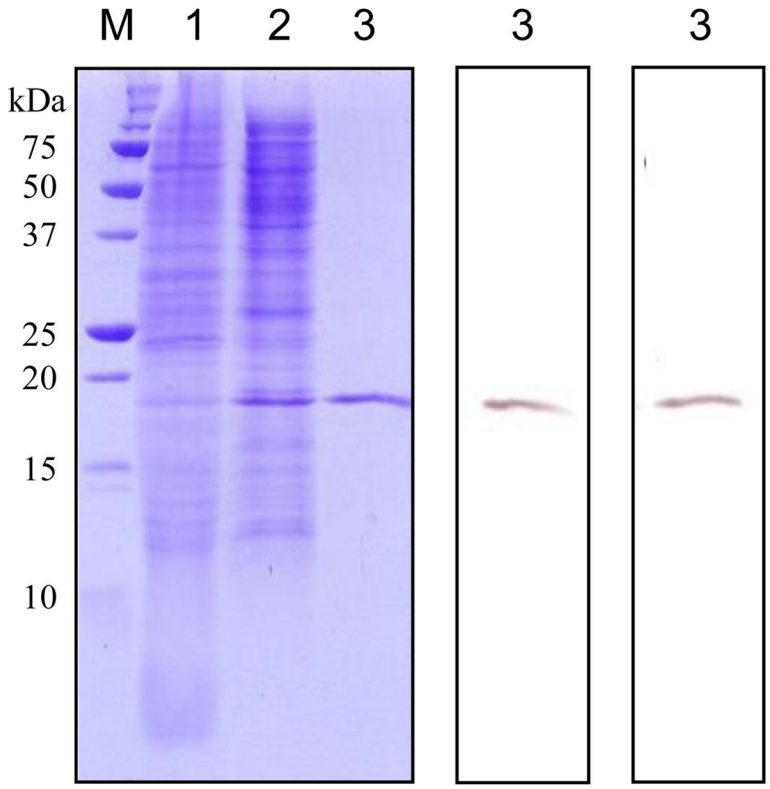

Fig. 3. Isolation of *M. sexta* PGRP1 from baculovirus-infected *Sf*9 cells

Left panel, Coomassie blue staining; central panel, immunoblot analysis using anti-$(His)_5$ as the first antibody; right panel, immunoblot analysis using diluted PGRP1 antiserum as the first antibody. Conditioned cell culture medium (lane 1, 10 μl), proteins eluted from dextran sulfate-Sepharose (lane 2, 10 μl), and affinity-purified PGRP1 from Ni^{2+}-NTA agarose (lane 3, 10 μl) were separated by 15% SDS-PAGE, along with pre-stained molecular weight standards (M) with their sizes indicated on the left.

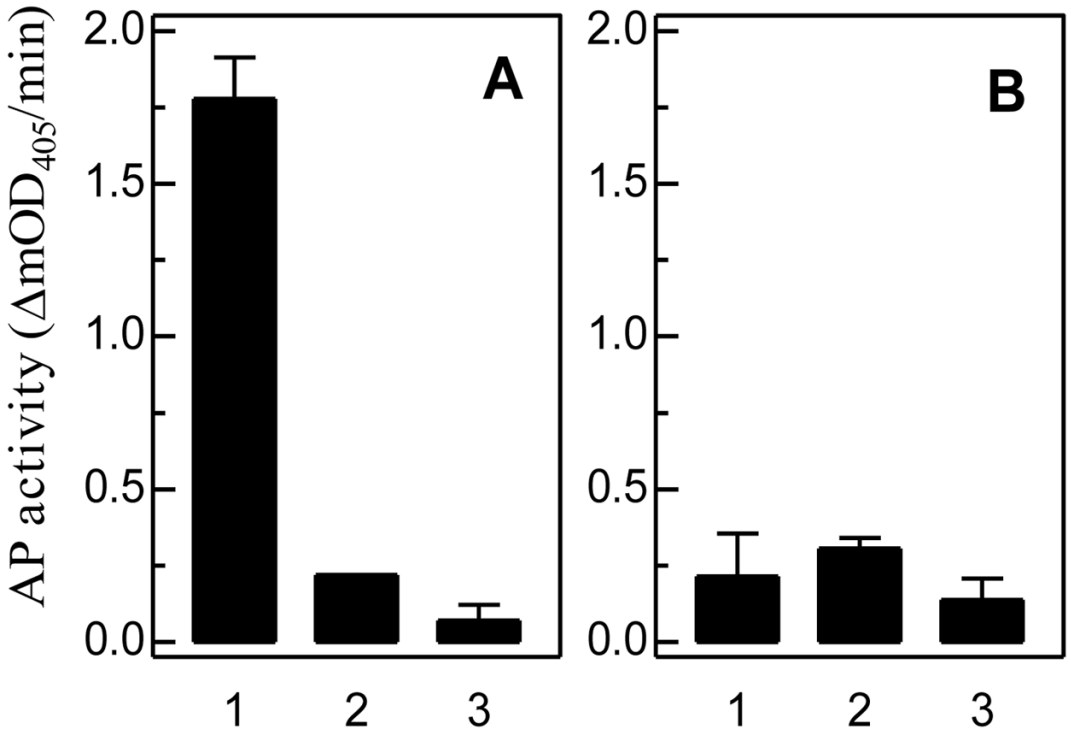

Fig. 4. Binding of *M. sexta* PGRP1 to soluble peptidoglycans from *E. coli* (A) and *S. aureus* (B)

As described in Section 2.6, the purified recombinant PGRP1 was incubated with soluble PG immobilized on a 96-well microplate. The binding was detected via ELISA and alkaline phosphatase activity is shown as mean ± SEM (*n*=3). Binding without a competitor (#1), with excess soluble PG as competitor (#2), and the negative control of BSA (#3).

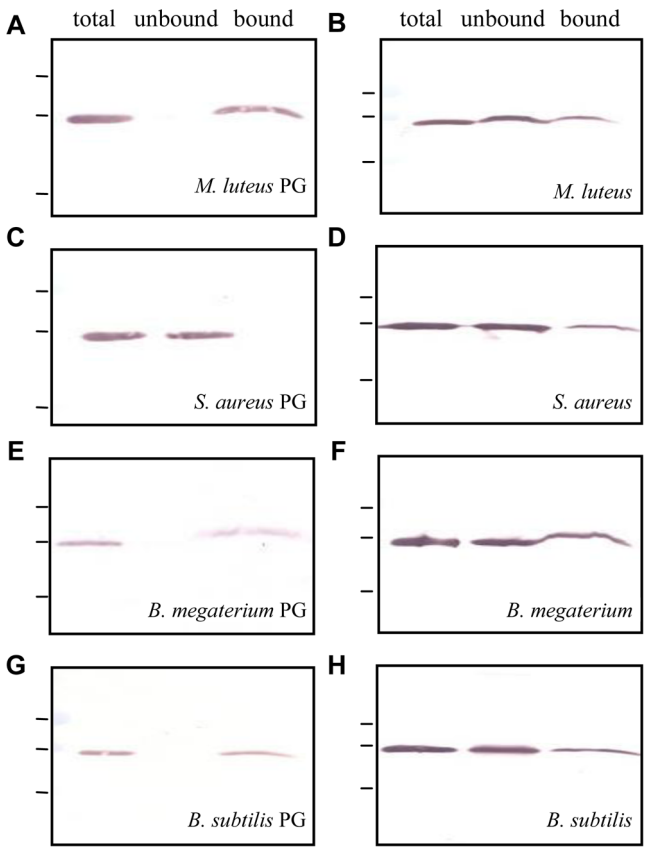

Fig. 5. Binding of *M. sexta* PGRP1 to insoluble PGs (A, C, E, G) or cells (B, D, F, H) of different bacteria

As described in Sections 2.8 and 2.9, binding assays were performed using the purified PGRP1 and PGs or whole bacteria. The total, unbound, and bound fractions were separated by SDS-PAGE followed by immunoblot analysis using anti-$(His)_5$ as the first antibody.

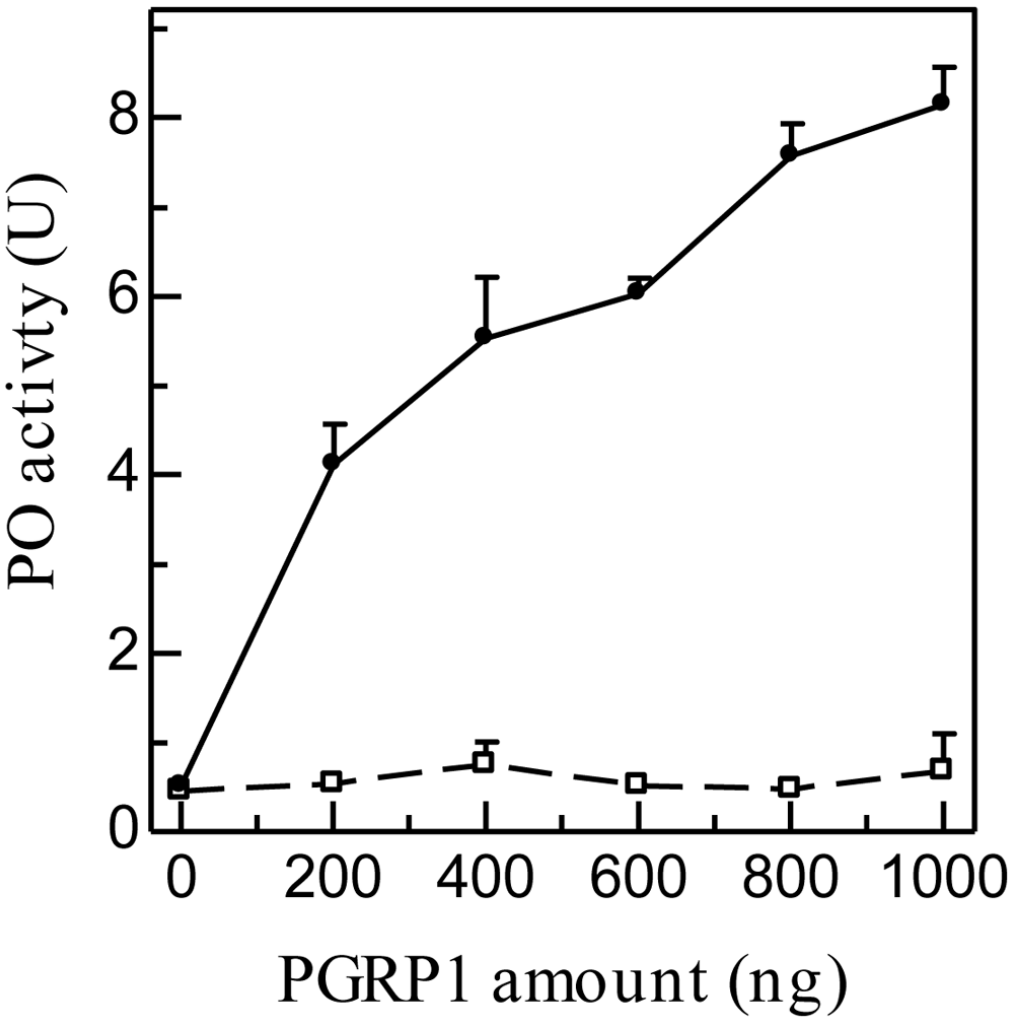

Fig. 6. PO activity increase in control hemolymph from naïve larvae after adding purified *M. sexta* PGRP1

Diluted plasma (5 μl) was incubated at room temperature with 0.2 to 1.0 μg of BSA (□ - - -□) or purified recombinant PGRP1 (■——■) in a final volume of 24 μl. PO activity was measured after 60 min and plotted as mean ± SEM (n = 3) against amount of PGRP1 added.

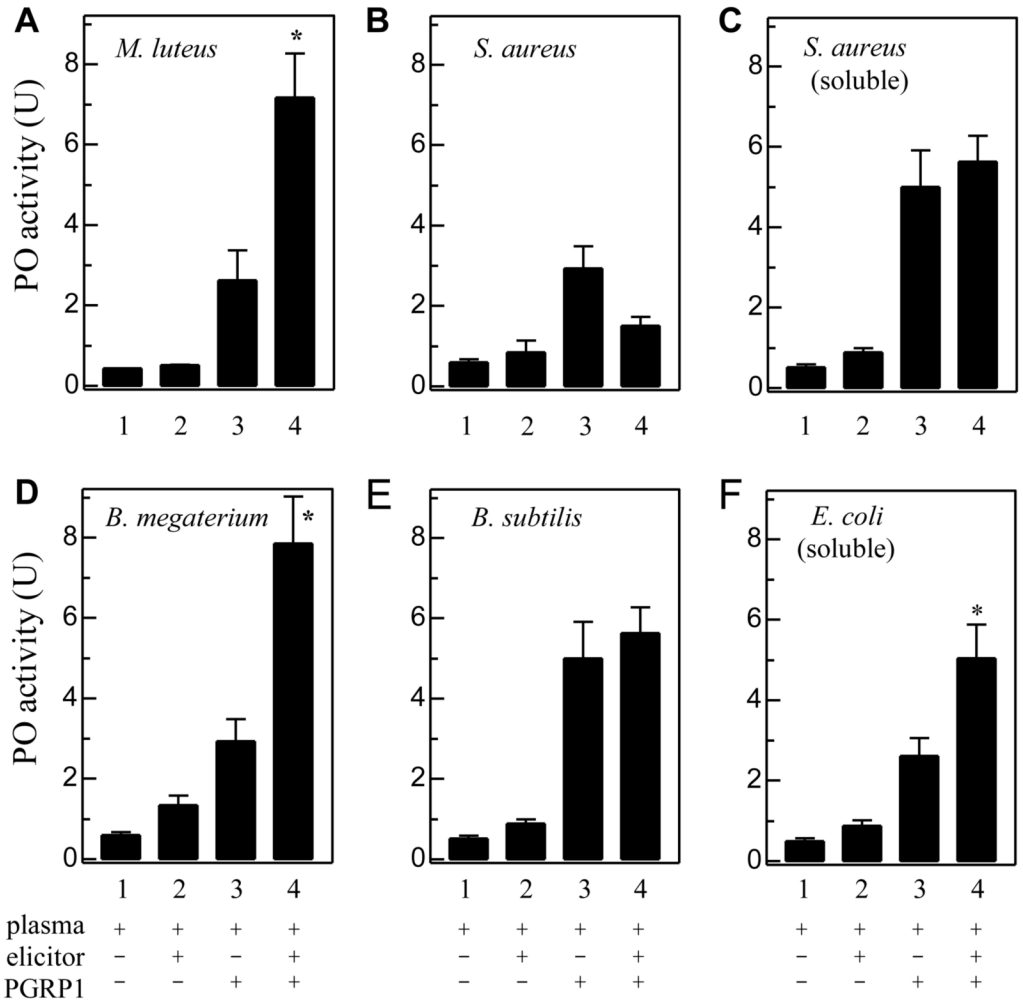

Fig. 7. Enhancement of proPO activation in plasma from naïve larvae by peptidoglacans in the absence or presence of *M. sexta* PGRP1

As described in Section 2.10, PGs of *M. luteus* (**A**), *S. aureus* (**B**), *S. aureus* (**C**, soluble), *B. megaterium* (**D**), *B. subtilis* (**E**), and *E. coli* (**F**, soluble) were separately incubated with plasma and purified PGRP1 for 60 min at room temperature. PO activity (#4) was measured and plotted as mean ± SEM (n = 3), along with those of the controls (#1, plasma only; #2, plasma and PG; #3, plasma and PGRP1). Since interaction of plasma factors with elicitor (#2 - #1) and co-presence of plasma and exogenous PGRP1 (#3 - #1) both lead to proPO activation, an interaction of elicitor and exogenous PGRP1 in plasma (#4 - #1) is expected to increase proPO activation to a level significantly higher than the sum of the two components [*i.e.* (#2 - #1) + (#3 - #1)]. To detect a possible synergistic effect of elicitor-PGRP1 interaction, the PO activity changes represented by (#4 - #1) and (#2 + #3 – 2×#1) are compared using unpaired t-test. An asterisk (*) on #4 indicates that (#4 - #1) is significantly higher ($p < 0.05$).

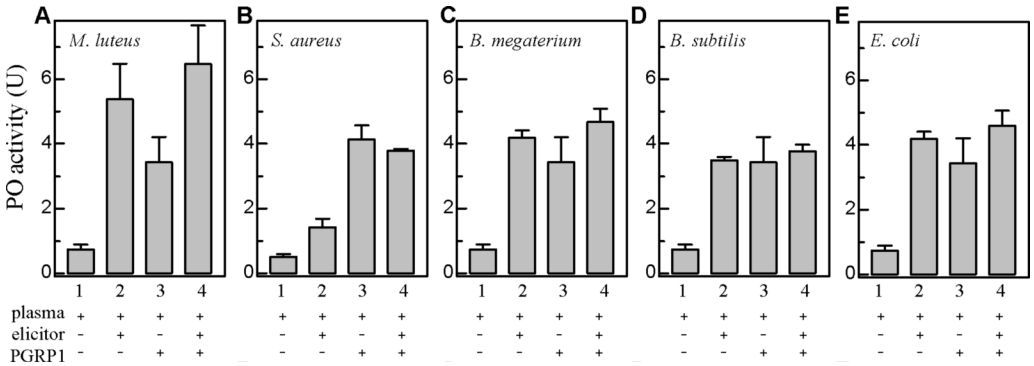

Fig. 8. Activation of proPO in plasma from naïve larvae by bacterial cells with or without *M. sexta* PGRP1

M. luteus (**A**), *S. aureus* (**B**), *B. megaterium* (**C**), *B. subtilis* (**D**), and *E. coli* (**E**) cells were individually incubated with plasma and purified PGRP1 for 60 min at room temperature. PO activity (#4) was measured and plotted as mean ± SEM (n = 3), along with those of the controls (#1, plasma only; #2, plasma and cells; #3, plasma and PGRP1). Since interaction of plasma factors with cells (#2 - #1) and co-presence of plasma and exogenous PGRP1 (#3 - #1) both lead to proPO activation, an interaction of bacteria and exogenous PGRP1 in plasma (#4 - #1) is expected to increase proPO activation at a level significantly higher than the sum of the two components [(#2 - #1) + (#3 - #1)] in an unpaired t-test. Comparisons of the PO activity changes, however, provide no support for any synergistic effect caused by bacteria-PGRP1 interaction.

Table 1

Relationship between binding of exogenous PGRP1 and increase in proPO activation

		Lys-type PG		DAP-type PG	
		M. luteus	***S. aureus***	***B. megaterium***	***B. subtilis***
bacterial cells	binding	*	*	*	*
	proPO activation	−	−	−	−
insoluble PG	binding	**	no	**	**
	proPO activation	+	−	+	+/−
soluble PG	binding	*S. aureus*	no	*E. coli*	**
	proPO activation		−		+

*partial binding;

**complete binding;

+ or −: synergistic enhancement of proPO activation.